10 Bioreactors for Tendon Tissue Engineering
Challenging Mechanical Demands Towards Tendon Regeneration

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10.1 INTRODUCTION

Tendons are unique connective tissues with the vital role to store and return elastic energy, resist damage, provide mechanical feedback and amplify or attenuate muscle power, and transmit forces from muscle to bone. Although tendon relevance in joint biomechanics and overall human body is often misunderstood and disregarded to other tissues, such as bone or cartilage, recent growing interest in tendon mechanical properties has highlighted potential studies working towards improved therapeutic strategies in the orthopedic field.

The term "tendon" comes from the Latin word, *tendere*, meaning to stretch (Liu et al. 2011). Despite the high tensile strength, tendons have limited intrinsic healing capabilities. It is estimated that approximately 50% of all musculoskeletal injuries are tendon-related (Praemer et al. 1992). Upon injury, the tendon undergoes degeneration and morphohistological misalignment of the collagen fibers. Ultimately, severe damage will result in pain and disability. Thus, a major challenge in tissue engineering (TE) and regenerative medicine is to recreate the tendon niche and replicate biomechanical forces involved in tendon functionality including stretching, loading, compression, and torsion.

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One potential approach to artificially generating the biomechanical demands of tendons is using complex advanced systems such as bioreactors. Bioreactors are designed considering the specific parameters of the replacing tissue or organ, especially in what concerns to tissue biomechanics and maintenance of a desired phenotype prior to implantation. Since the birth of TE in the early 1990s, bioreactors have been primarily used for studying basic pathways, expand and grow tissue/organ substitutes, maintain *ex vivo* organ vitality and priming therapeutic cells before implantation. The use of suitable biomechanical and biophysical environments in which cells could synthesize a functional matrix results in a closer mimicry of tendon tissue, leading to maturation of cell-laden constructs prior to implantation *in vivo.* This way, the utilization of bioreactors as *in vitro* models is expected to minimize the number of animal experiments as the implantation step only occurs when the morphological, biological and biomechanical properties of the engineered construct match those of the natural tissue.

In the tendon scenario, bioreactors have been used to culture tendon engineered substitutes and to investigate suitable *in vitro* conditions for establishing benchmarks and protocols for effective cell programming toward the tenogenic phenotype. Thus, bioreactors are promising tools for developing and culturing *in vitro* generated tendon substitutes, as potential alternatives to pharmacological therapies and to fulfill the current need for tissue substitutes to treat tendinopathies.

This chapter will outline state of the art TE strategies on cell culture or cell laden 3D matrices using mechanically active environments provided by bioreactor systems for tendon regeneration as a potential means to obtain functional tendon substitutes. For a better understanding on the performance of these systems and their role in strategies applied to tendon TE, the intrinsic properties and requirements of tendons will be explored with an emphasis on the role of biomechanical stimulation in tendon development and maturation, as well as the biomechanics-tissue functionality relationship.

10.2 TENDON STRUCTURE-FUNCTION AND MECHANOBIOLOGY BEHAVIOR

10.2.1 Role of Mechanical Stimulation in Tendon Development and Functionality

Tendons are specialized connective tissues that serve to transmit forces between muscles and bones, and thus allow body motion. Their crucial role in musculoskeletal functionality implies distinct mechanical properties, which are assured by tissue-specific structure and molecular composition, namely highly organized collagen fibers arranged parallel to the tendon axis. The smallest structural unit of tendon is fibril composed of collagen molecules assembled in a quarter-staggered D-periodic pattern (Kastelic et al. 1978). Fibrils form fibers, which group together to form fiber bundles or fascicles, enveloped by thin layer of connective tissue called endotenon. Fascicles bundles are, in turn, enclosed by another layer of loose connective tissue sheath, the epitenon, that provides vasculature, lymphatics, and innervation to the tendon unit. Tendons may be eventually enveloped by aleoral sheath of paratenon AQ 2
that serves to reduce friction with adjacent tissues, thus enabling free tendon movement against its surroundings (Kastelic et al. 1978).

Tendon fibroblasts- tenocytes- are found longitudinally aligned in the rows AQ 3
between collagen fibers, and are mainly responsible for the synthesis and maintenance of the extracellular matrix (ECM). Interestingly, a population of cells with universal stem cell characteristics, named *tendon stem/progenitor cells* (TSPCs), has been identified in both human and murine tendons (Bi et al. 2007).

The major component of tendons is collagen type I that represents approximately 95% of the total collagen content and around 60% of the tissue dry mass (Riley et al. 1994). Tendon-specific mechanical integrity and function is acquired through a multistep process of collagen fibrillogenesis during tendon development (Birk and Zycband 1994, Silver et al. 2003, Zhang et al. 2004). In the first stage, collagen molecules assemble in the extracellular space to form immature fibril intermediates. Fibril intermediates associate subsequently end-to-end forming longer and mechanically functional fibrils. The linear growth is then followed by a lateral growth step, where fibrils associate laterally generating fully mature fibrils with larger diameters (Zhang et al. 2004). The process of fibril assembly is regulated by heterotypic interactions between fibril-forming and fibril-associated collagens, and fibril-associated proteoglycans. For example, the interaction between two fibrillar collagen type I and type III plays a role in initial fibril assembly and control of fibril diameters (Banos et al. 2008). The ratio of collagen type III to collagen type I exerts spatial and temporal variations throughout tendon development. On the other hand, in the mature tendon, collagen type III is present mainly in the endotenon and epitenon. Similarly, collagen type V may assemble with collagen type I and has been implicated in fibril nucleation and diameter regulation (Birk et al. 1990, Wenstrup et al. 2004). Type XII and type XIV collagens, which represent Fibril-Associated Collagens with Interrupted Triple-helices (FACITs), are localized near the surface of fibrils and may contribute to fibrillogenesis regulation by providing molecular bridges between collagen fibrils and other components of

the ECM. Although their role in fibril assembly is not well understood yet, it was hypothesized that collagen type XII may stabilize fibril structure during tendon development (Chiquet 1999), while type XIV limits fibril diameter (Young et al. 2002). Beside the collagen class, molecules that belong to the family of small-leucine-rich proteoglycans (SLRPs), such as decorin, biglycan, fibromodulin, and lumican, are believed to actively regulate tendon fibrillogenesis, since their targeted disruption in mouse models lead to abnormal fibril phenotypes (Vogel and Heinegård 1985, Yoon and Halper 2005, Subramanian and Schilling 2015). Interestingly, biglycan and fibromodulin have been recognized as critical components of tendon stem cell niche regulating TSPCs differentiation and tendon formation *in vivo* (Bi et al. 2007).

Noteworthy, the cellular composition and collagen organization are not uniform along the tendon length and demonstrate regional differences in the myotendinous junction, which is the interface between tendon and muscle and in the tendon to bone attachment site, called enthesis (Thomopoulos et al. 2003). These molecular and cellular variations are translated in different mechanical properties of specific tendon regions that reflect nonhomogeneous mechanical loading requirements in different anatomical sites (Genin et al. 2009).

The molecular mechanism governing the synthesis and spatial organization of collagen in developing tendons has not been fully elucidated. Since collagen is the main component of various connective tissues, it is impossible to trace tendon development by mapping its expression. In fact, no marker unique for tendons has been identified to date. The basic helix-loop-helix transcription factor scleraxis (Scx) has been described as an early tendon marker, whereas a type II transmembrane glycoprotein tenomodulin (Tnmd) is considered a late tendon marker (Shukunami et al. 2006). Though not specific to tendon, two other transcription factors are involved in tendon development, the homeobox protein Mohawk (*Mkx*) and a member of zinc finger transcription factor family, early growth response factor 1 (Egr1). *Mkx*-null mice presented a wavy-tail phenotype and hypoplastic and less vibrant tendons throughout the body with reduced fibril diameters and down-regulation of type I collagen expression, when compared to the wild type (WT) counterparts. In addition to disruption of postnatal collagen fibrillogenesis, mutant mice exhibited abnormal tendon sheaths (Ito et al. 2010, Liu, Watson et al. 2010). Similarly, Egr1 has been shown to positively regulate collagen transcription in postnatal tendons. *Egr1*$^{-/-}$ mutant mice demonstrated a deficiency in expression of *Scx*, *Col1a1* and *Col1a2* genes, reduced fibril diameter and packing density, resulting in mechanically weaker tendons, compared with their WT littermates (Lejard et al. 2011, Guerquin et al. 2013). Interestingly, ectopic expression of either *Mkx* or *Egr1* promoted tenogenic differentiation of mesenchymal stem/stromal cells (MSCs) via activation of (Transforming growth factor beta) TGF-β signaling pathway (Guerquin et al. 2013, Liu et al. 2014).

Tendon embryogenesis has not been fully investigated, and most of the data comes from developmental studies in invertebrates and chick and mouse models. The vertebrate tendons originate from mesoderm or mesectoderm, more

specifically, the craniofacial tendons are derived from neural crest cells, axial tendons originate from syndetome, whereas limb tendons come from the limb lateral plate. Notably, tendons share same embryological origins with cartilage and bone, but not with skeletal muscles, which originate from dermomyotome (axial), mesoderm (head), or somites (limb). Despite these distinctions, the development of various components of the musculoskeletal system progresses in their close spatial and temporal association. It has been demonstrated that depending on the anatomic location, tendon development requires the presence of muscle. In chick somites, surgical ablation of dermomyotome prior to myotome formation results in the absence of *Scx* expression, indicating that muscle is required for initiation of development of axial tendons (Brent et al. 2003). Similarly, in *Myf5*$^{-/-}$*; MyoD*$^{-/-}$ double-mutant embryos *Scx* expression is undetectable in mouse somites, and further supports the fact that myotome specification is indispensable for axial tendon progenitor formation (Brent et al. 2005). Contrarily, limb and head tendon development are initiated independently of muscles in mouse, chick, and zebrafish embryos. *Scx* expression is induced normally in muscleless limbs of *Pax3* mutant mice (Schweitzer et al. 2001) and *myod1-myf5* deficient zebrafish embryos, as well as in murine and zebrafish craniofacial tendons (Berthet et al. 2013). However, eventually the absence of muscles results in tendon development arrest and loss of *Scx* expression (Schweitzer et al. 2001, Bonnin et al. 2005). Hence, muscle is crucial for *Scx* induction in axial tendons, as well as for the maintenance of its expression in cranial and limb tendons. Since this pattern has been conserved across different species, it may indicate a requirement for mechanical forces provided by muscle during tendon morphogenesis. The two main signaling pathways identified as being involved in tendon development are TGFβ-SMAD2/3 and Fibroblast growth factor (FGF)-ERK/MAPK pathways (Havis et al. 2014). FGF signaling from the myotome was first associated with the induction of *Scx*-expressing tendon progenitors in adjacent somatic subcompartment of developing axial tendons in chicks (Brent et al. 2003). Disruption of TGFβ signaling in *Tgfb2*$^{-/-}$ and *Tgfb3*$^{-/-}$ double-mutant embryos leads to the loss of most tendons and ligaments (Pryce et al. 2009). Since *Scx* expression is disrupted only at E12.5, it has been suggested that TGFβ is required for tendon progenitor maintenance. Interestingly, in pharmacologically immobilized chick embryos both FGF and TGFβ signaling cascades were downregulated, suggesting that FGF and TGFβ ligands regulate tendon differentiation acting downstream to mechanical forces present in developing embryos (Havis et al. 2016). Additionally, growth differentiation factors (GDFs) that belong to the bone morphogenetic protein (BMP) family have been implicated in tendon development. *GDF-5* deficient mice exhibited altered ultrastructure and composition and inferior mechanical properties of Achilles tendon, when compared with control littermates (Mikic et al. 2001). Similarly, *GDF-6* deficiency in mice was associated with reduction in tail tendon collagen content and compromised tail material properties (Mikic et al. 2009). Beside those mentioned thus far, some other signaling pathways, such as the highly conserved Wnt pathway or calcium signaling might be involved in tendon morphogenesis.

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10.3 BIOMECHANICS IN HOMEOSTASIS AND TENDINOPATHIC TENDONS

Being subjected to dynamic mechanical forces *in vivo*, tendons exhibit a unique crimp pattern and viscoelastic properties akin to a spring that enable tendon to effectively store and subsequently release mechanical energy. In a typical tendon stress-strain curve, four different regions can be distinguished. The initial toe region, where tendon is strained up to 2%, corresponds to the stretching-out of the characteristic crimp pattern. In the linear region of the curve, where stretching does not exceed 4%, collagen fibers lose their crimp pattern; the slope of the linear region defines the Young's modulus (i.e., elasticity) of the tendon. Stretching over 4% results in microscopic tearing, whereas strain beyond 8%–10% leads to macroscopic failure and tendon rupture (Wang 2006). Studies of force-length relationship revealed that with increasing forces, tendons lengthen to a certain degree (ascending limb)- after a certain point application of force results in tendon failure (descending limb) (Maganaris et al. 2004). Viscoelastic properties of tendon are defined by creep, that indicates increasing deformation under constant load, stress relaxation upon deformation, as well as hysteresis, or energy dissipation, which implies that an amount of energy is lost during loading. Consequently, the loading and unloading curves look differently. Mechanical properties vary depending on tendon anatomical site and specific function and are therefore dictated by the level of mechanical load to which a particular tendon is subjected (Bennett et al. 1986). These mechanical forces placed on tendons are, in turn, determined by the type of activity, passive or active mobilization, joint position, level of muscle contraction, tendon relative size, and so on. Additionally, variations in the rate and frequency of mechanical loading would result in different tendon forces (Wang 2006).

10.3.1 Loading and Overuse

Tendons are metabolically active tissues and tendon-resident fibroblasts respond to dynamic mechanical loading by alterations in the synthesis of ECM components and matrix degrading enzymes. A growing body of evidence supports the key role of mechanical stress in promotion and maintenance of tendon-specific phenotype. While mechanical forces are essential for tendon development and homeostasis, both complete unloading and contrarily excessive loading beyond a physiological range might have detrimental effects on tendon functionality.

Hannafin and colleagues investigated the effect of stress deprivation and cyclic tensile loading on histological and mechanical characteristics of the canine flexor digitorum profundus tendon (Hannafin et al. 1995). Stress deprivation resulted in significant changes in cell morphology and number, collagen fiber alignment, and progressive decrease in the tensile modulus over an eight-week period. However, tendons subjected to cyclic tensile loading for 4 weeks demonstrated increased Young's modulus (93% of the control) when compared to stress-deprived tendons (68% of the control), as well as maintained normal histological patterns (Hannafin et al. 1995). Surgical release of tensile strain in an engineered human tendon model resulted in

disruption of tendon architecture, downregulation of tendon-related markers and induction of pro-inflammatory mediators (Bayer et al. 2014). To determine if the loss of tensile tension could induce apoptosis in tendon cells, Egerbacher and co-workers cultured rat tail tendons for 24 hours under cyclic loading or stress-deprived conditions. Upregulated caspase-3 expression and the increased number of apoptotic cells in stress-deprived tendons, when compared with the loaded group, indicated that loss of homeostatic tension induces programmed cell death (Egerbacher et al. 2008). Employing a transgenic mouse model, where GFP expression is driven by AQ 7
the *Scx* promoter, Maeda and colleagues demonstrated that gradual and temporary loss of transmittal forces from skeletal muscles by application of botulinum toxin A resulting in reversible loss of *Scx* expression and a decline in tendon mechanical properties. Acute loss of tensile loading by complete tendon transection led, in turn, to destabilization of the ECM structure, excessive release of active TGF-β and massive tenocyte death (Maeda et al. 2011).

Wang and collaborators investigated the effect of different mechanical stimulation regimes on rabbit Achilles tendon integrity in a bioreactor system (Wang et al. 2013). In the absence of loading, gradual loss of collagen fiber organization, increased cellularity, and cell roundness were observed, indicating a progressive divergence from the native tendon phenotype. Tendons stimulated with 3% cyclic tensile strain demonstrated moderate ECM disruption and elevated expression levels of matrix metalloproteinases (MMPs), MMP-1, -3 and -12, whereas excessive loading of 9% resulted in partial tendon ruptures. However, tendons stimulated with 6% cyclic tensile strain maintained their structural integrity and cellular function, suggesting that there is a narrow range of tensile loading promoting an anabolic effect and tendon tissue homeostasis (Wang et al. 2013). In a follow-up study, the model was extended to characterization of degenerative changes observed in tendons under loading-deprived conditions. When unloaded for 6 and 12 days, tendons exhibited progres- AQ 8
sive degenerative alterations, abnormal collagen type III production, increased cell apoptosis, and impaired mechanical properties. However, the application of a 6% cyclic tensile strain at day-7 for another six days was able to reverse morphological degenerative changes and partially restore mechanical properties of the unloaded tendon to the levels characteristic for the healthy tissue (Wang et al. 2015). Although mechanical stimulation is crucial for tendon-specific phenotype maintenance, excessive mechanical loading has been implicated as the major causative factor of tendon overuse injuries, collectively referred to as tendinopathies.

Histopathological presentation of painful tendons may comprise increased or decreased cellularity, cell rounding, increased vascularity and innervation, increased collagen type III expression and proteoglycans content, and collagen fibril disorganization. Molecular changes in tendinopathy include elevated expression of collagen type I and III, biglycan, fibromodulin, aggrecan, fibronectin, tenascin C (TNC) and alterations in expression levels (both upregulation and downregulation) AQ 9
of MMPs, and tissue inhibitor of metalloproteinases (TIMPs) that regulate ECM turnover (Corps et al. 2006, Jones et al. 2006). However, the etiology of tendon injuries has not been fully elucidated yet, and especially the role of inflammation in tendon pathology and healing process remains the subject of debate and ongoing

controversy (Riley 2008, Dakin et al. 2015, Dean et al. 2016, Millar et al. 2017, Fredberg and Stengaard-Pedersen 2008). Several factors have been postulated to be implicated in tendon disease occurrence, including age, gender, body weight, vascular perfusion, nutrition, joint laxity, systemic diseases, muscle weakness, physical load, repetitive loading, abnormal movement, poor technique and training errors, fast progression and high intensity, environmental conditions, running surface, and more. Moreover, genetic predisposition (e.g., variants within COL5A1, TNC-C, and MMP3 genes in Achilles tendinopathy), treatment with corticosteroids or fluoroquinolones, oral contraceptives uptake, as well as existing comorbidities, such as obesity, diabetes, or hyperlipidemia, have been proposed as risk factors in tendon pathology development (Fredberg and Stengaard-Pedersen 2008, Magra and Maffulli 2005, 2008, Maffulli et al. 2013).

Soslowsy and colleagues employed an intensive running regime for 4, 8, and 16 weeks to induce an overuse injury in a rat model. Compared to the control group, which was allowed normal cage activity, the supraspinatus tendons in the exercised animals demonstrated increased cellularity and collagen fiber disorganization, the features that are normally observed in human tendinopathy. The tendons from the running group exhibited enlarged cross-sectional area and decreased mechanical properties, when compared to the control group (Soslowsky et al. 2000). In an *in vitro* study by Thorpe and co-workers, the application of cyclic loading mimicking high intensity exercise resulted in matrix damage and cell rounding in equine superficial digital flexor tendon explants. Those morphological changes were accompanied by increased expression of inflammatory mediators and MMPs in the loaded samples, when compared to the control group (Thorpe et al. 2015). Similar ECM damage and inflammatory response was observed in bovine flexor tendon overloading model (Spiesz et al. 2015).

Due to low cellularity, poor vascularization and innervation, tendons demonstrate restricted intrinsic healing capacity. A repaired tendon never regains the mechanical properties and hence full functionality of the pre-injured tissue, indeed, final tensile strength of healed tendon might be reduced by up to 30% (Majewski et al. 2008) or not restored two years after surgical repair (Geremia et al. 2015). After an acute injury, the tendon healing process normally follows a course of distinct, overlapping stages of early inflammation, proliferation, and remodeling, each orchestrated by a specific set of cellular and biochemical components. In the initial inflammatory phase, erythrocytes, platelets, and inflammatory cells (e.g., neutrophils, monocytes, macrophages) infiltrate the wound site and release vasoactive and chemotactic agents to promote angiogenesis and fibroblasts recruitment. During the proliferative phase, tendon fibroblasts synthesize collagen and other ECM components leading to granulation tissue formation around the wound site. After 6–8 weeks, final remodeling phase commences. This stage is characterized by decreasing cellularity and reorganization of collagen architecture where collagen type III is replaced by collagen type I. As the scar matures, covalent bonding between collagen fibers increases, which leads to higher tendon stiffness and tensile strength. Yet, the healed tendon never matches characteristics of intact tissue. During tendon healing upregulation of several growth factors and cytokines, such as insulin-like growth factor-1 (IGF-1),

platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β), stimulate cell migration, proliferation, angiogenesis and synthesis of collagen and other ECM components (Voleti et al. 2012, Millar et al. 2017).

Tendon stem/progenitor cells role in tendon homeostasis and disease is not well understood yet, however it was suggested that TSPC malfunction may contribute to impaired healing and repair, or tendon pathology development. Especially, age-related depletion of the stem cell pool and/or a decline in stem cell function associated with entrance of senescence state might be implicated in pathology onset and progression. The mechanoresponse of TSPCs have been studied both *in vitro* and *in vivo* indicating a critical role of mechanical loading in tendon stem cell fate and function. Mechanical loading at physiological level (4% stretching) promoted TSPCs proliferation and differentiation into tenocytes, whereas at excessive stretching (8%) TSPCs differentiated in non-tenocytes such as adipocytes, chondrocytes and osteocytes, in addition to tenocytes (Zhang and Wang 2010a). In a follow-up *in vivo* study employing mouse treadmill running model it was found that tendons subjected to repetitive, strenuous mechanical loading produced high levels of PGE_2, which in turn was associated with decreased proliferation of TSPCs and TSPC differentiation into adipocytes and osteocytes (Zhang and Wang 2010b). Such non-tenocyte differentiation of TSPCs under abnormal mechanical loading may explain some pathological features of late tendinopathy such as lipid accumulation, mucoid formation and tissue calcification.

Management of tendon injuries includes surgical procedures and nonsurgical modalities such as physiotherapy. Application of mechanical stimulation may be beneficial for the proper organization of collagen fibers and prevention of adhesion formation during tendon healing. In injured canine flexor tendons, active mobilization increased their tensile strength and restored gliding surfaces while reducing intrasynovial adhesion formation (Gelberman et al. 1986, Wada et al. 2001). In a rabbit model of Achilles tendon healing, early mobilization after tenotomy favored a more rapid restoration of tissue functionality, when compared to the group subjected to continuous immobilization (Pneumaticos et al. 2000). A study of 64 human patients with Achilles tendon ruptures treated surgically and with early mobilization indicated that application of an early mobilization rehabilitation program reduces the range of motion loss and muscle atrophy, increases blood supply, as well as improves strength of calf the muscles and ankle movement (Sorrenti 2006). 12 weeks of eccentric resistance training in elite soccer players increased peritendinous type I collagen synthesis in individuals suffering from Achilles tendinosis, whereas collagen metabolism was not affected in the healthy control group (Langberg et al. 2007). However, a 10-year follow up study of postoperative regimes of Achilles tendon ruptures showed that early mobilization and immobilization in tension resulted in similar clinical outcomes and isokinetic strengths (Lantto et al. 2015). Although some conflicting data exists and the optimal rehabilitation protocol and precise molecular mechanism underlying the beneficial effects of mobilization remain to be determined, controlled tendon-loading and motion plays crucial role in tendinopathy management (Rees et al. 2006).

10.4 CURRENT THERAPIES FOR THE MANAGEMENT OF TENDINOPATHIES

Conservative treatments and/or grafting surgeries are the gold standards for the treatment of tendon injuries. The treatment of choice is influenced by tendon location, type and severity of lesion as well as on the symptoms and clinical evidence of injury.

Independently of the treatment selected, the mid to long-term outcomes are not completely satisfactory with a risk of recurrence of symptoms that include pain, instability and degradation of mechanical function.

In the case of tissue grafting, besides the morbidity of the donor tissue and the risk of (re)rupture of the inflicted tendon, both tendons may experience long-term consequences as loss of mechanical competence, functional disability and degeneration that may progress into nearby tissues.

Alternatively, tissue grafting from autologous or cadaveric sources, biological augmentation matrices of decellularized mammalian-origin tissues mainly human (GraftJacket®), porcine (Restore™), equine (OrthADAPT®) or bovine (TissueMend®) have been investigated and presented to the clinical field, revised by Chen et al (Chen et al. 2009). The main reasons for a lack of compliance on the medical use of these devices may be caused by the decellularization process that may be insufficient to remove all the resident cells and there is a potential risk of immune-rejection and for zoonoses transmission.

Artificial augmentation devices constitute an alternative to tissue grafting procedures and to biological augmentation devices (Liu et al. 2010). Commercially available devices as LARS™, Kennedy ligament augmentation device, Dacron®, Gore-Tex and Trevira, revised by Batty and colleagues (Batty et al. 2015), were described to avoid and provide improved knee stability (Liu et al. 2010) and full weight bearing. However, artificial devices have shown controversial outcomes on the long-term follow up, concerning mechanical failure or mechanical mismatch with native tissues, instability, synovitis, chronic effusions and progression to early osteoarthritis.

10.5 TENDON TISSUE ENGINEERING STRATEGIES

Tendons require a unique combination of cells within an abundant, hierarchically organized ECM coordinated by mechanical, biochemical, and architectural sensing and signaling. A failure to this balance results in significant non-functional modifications and/or disease.

A traditional TE strategy is inspired by the natural elements within a tissue niche, namely cells, a 3D structure and their highly orchestrated biochemical signaling in different combinatorial approaches with the final goal of stimulating and inducing new tendon formation with restored function (Figure 10.1). As mechano-responsive tissues, mechanical conditioning of tendons is essential and a critical parameter of the native environment for tissue development and maturation, which ultimately will translate into successful 3D tissue equivalents and improved clinical therapies (Figure 10.1).

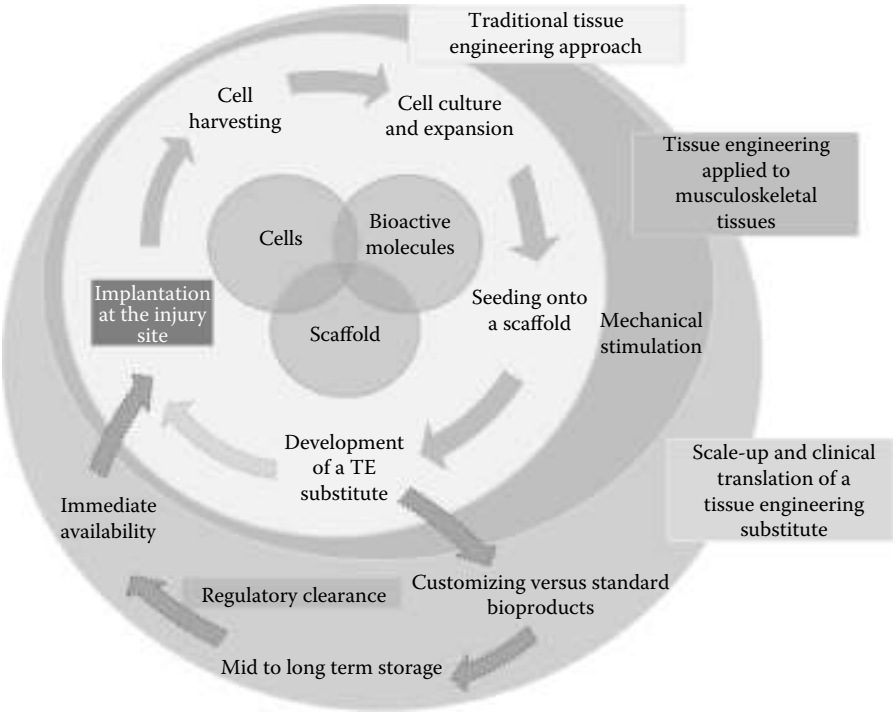

FIGURE 10.1 Diagram on the development of a successful tissue engineered substitute towards clinical translation using a traditional TE approach by employing bioreactors.

10.5.1 The Role of Cells in Tendon Tissue Engineering

Tendon resident cells, mainly tenoblasts and tenocytes, are scarce and are responsible for the maintenance of ECM. These elongated cells stretched between the collagen fibers of the tendon, synthesize collagen and other macromolecules, assemble these molecules into a cohesive unit, and organize the fiber phase in parallel with the direction of tensile load, which is capable of transducing and responding to mechanical stimuli (Koob 2002). This organization makes it extremely difficult to repair damage to tendinous structures. Natural tendon healing usually leads to the formation of fibrotic tissue, impairing tendon mechanical properties, and resulting in a poor quality of life. More recently, a tendon stem/progenitor cell population was identified (Bi et al. 2007) and described to participate in the endogenous regeneration process of tendons.

The hypocellular and hypovascular nature of tendons can relate to significant limitations in the process of repair and regeneration, especially upon tears and ruptures. Cells, especially the resident cell populations, have a critical role in the regulation of the tendon matrix in both normal and pathological conditions (Clegg et al. 2007) being an important parameter when considering engineering the functional tendon inspired substitutes.

Several cell sources have been investigated for tendon strategies (Gonçalves et al. 2015). Tendon cells are often the first choice despite the donor tissue morbidity and the infliction of a secondary defect to heal the damaged tendon. However, they are still a great source to study mechanisms and predict native biological responses. Other sources, such as dermal fibroblasts or muscle-derived cells (Chen et al. 2016), have been also exploited as cell alternatives for tendon approaches.

Stem cells are also a promising source due to their high self-renewal potential and ability to differentiate into tissue-oriented lineages, with evidence to commit toward a tenogenic fate (Gonçalves et al. 2013, 2017). The secretion of a broad range of bioactive molecules with paracrine effects is believed to be the main mechanism by which MSCs achieve their therapeutic effect (Meirelles et al. 2009). The fact that tendons have their own local stem cell population may indicate that stem cells are likely to participate and even mediate the renewal and remodeling of tissues, but also influence fibrous scarring due to abnormal or irregular signaling mechanisms and influence tissue recovery upon injury.

More recently, embryonic stem cells (ESCs) (Cohen et al. 2010, Chen et al. 2012) and induced pluripotent stem cells (iPSCs) technology (Xu et al. 2013, Bavin et al. 2015) have also been proposed for tendon tissue engineering (TTE). Ethical considerations associated with human embryos research and the risk of *in vivo* teratoma formation presents significant drawbacks for the clinical application of ESCs. A potential strategy to overcome many current ethical concerns in ESCs-based therapy is the use of iPSCs (Docheva et al. 2015), which are highly available cells obtained from multiple sources of autologous cells and can be reprogrammed into a wide range of cell types. The very few studies on human iPSCs differentiated for tendon applications (Xu et al. 2013, Bavin et al. 2015) show controversial outcomes and their potential for tendon fundamental studies and clinical approaches should be more deeply investigated.

Apart from cell sources, cell-based therapies offer the potential to induce a regenerative response by stimulating local cells and inspiring the synthesis of a structural matrix to ensure remodeling of damaged tendon tissues.

10.5.2 Bioactive Molecules in Tendon Engineered Substitutes

The lack of understanding on cell-to-cell and cell-matrix communication in tendon niches results in limited knowledge to guide biological responses for effective treatment. Moreover, cell secretome and signaling interactions are envisioned to represent the most biologically significant cell role towards repair mechanisms.

Several biomolecules, some identified in developmental biology studies and discussed earlier in this chapter, have been described to participate in tenogenic commitment, namely EGF, FGF, PDGF, IGF-1, GDF-5, TGF-β (Gonçalves et al. 2013, Barsby and Guest 2013, Holladay et al. 2014, Bottagisio et al. 2017); however, their precise spatio-temporal distribution requires further research to promote *in vitro* tenogenic differentiation. Thus, progress towards the recognition of the molecular players favoring homeostasis and regeneration will assist the establishment of tendon benchmarks and methodologies paving the way for clinical translation of cell-based therapies.

10.5.3 Recapitulating Tendon Matrix in Functional Substitutes

The matrix of tendon tissues is quite unique and complex: it complies with a supportive function of cells as its organization and structure relates to tendon mechanical properties and function adapted to the anatomical location.

Thus, another main challenge in the successful design of a tendon substitute is to mimic the intrinsic alignment of tendons and recapitulate their complex hierarchical architecture, while remaining mechanically competent towards achieving proper biomechanical functionality to support a complete regeneration of damaged tissues.

Leading edge biomaterial advances have featured fiber fabrication technologies to produce architecturally aligned scaffolds aiming at tendon replacement strategies. These technologies include 3D-printing (Goncalves et al. 2016), a combination of polyelectrolyte complexation and microfluidics (Costa-Almeida et al. 2016, Shilpa 2017), electrospinning (Shuakat and Lin 2014, Wang et al. 2015), and an electrochemical alignment technique (Gurkan et al. 2010, Younesi et al. 2014) combined with textile techniques.

A magnetic responsive scaffold based on a polymeric blend of starch and polycaprolactone (PCL) fibers (SPCL) with aligned structural features incorporating magnetic nanoparticles by 3D-printing was shown to assist the tenogenic differentiation of human adipose-derived stem cells (hASCs) upon the actuation of an external magnetic field and evidenced good biocompatibility and integration within the surrounding tissues upon implantation in an ectopic rat model (Goncalves et al. 2016).

Instructive tenogenic matrices with microscaled parallel aligned fibrils were developed by combining polyelectrolyte complexation and microfluidics (Costa-Almeida et al. 2016, Shilpa 2017). These fibrous photocrosslinkable hydrogels were fabricated with chitosan (positively charged) and methacrylated gellan gum (negatively charged) (Sant et al. 2016), a combination of alginate (ALG) with methacrylated hyaluronic acid (MeHA, negatively charged), or chondroitin sulfate (Costa-Almeida et al. 2016). The MeHA-ALG fibers could be manipulated using textile technologies, allowing the fabrication of 3D constructs with increasing complexity and functionality.

Electrospinning is a familiar technique to TE approaches. It allows the production of long and continuous fibers with controlled diameter mimicking the ECM of the tendon at the nanoscale. Nano-scaled fibers are expected to provide topographical cues at the cell level and stimulate cell response by contact. Accordingly, several studies report that aligned nanofibers can stimulate different cell sources, including dermal fibroblasts (Wang et al. 2016) and iPSCs (Zhang et al. 2015) to commit towards the tenogenic phenotype (Wang et al. 2016) in both synthetic and natural based scaffolds (Teh et al. 2013, Zhang et al. 2015, Wang et al. 2016, Sensini et al. 2017) and to enhance tendon regeneration *in vivo* (Wang et al. 2016). However, nanofiber scaffolds produced by electrospinning are mainly 2D systems and their scaling up into 3D scaffolds is limited unless combined with other scaffolding fabrication techniques (for instance rapid prototyping or textile approaches such as braiding and weaving).

The advancement of medical textiles has created a new generation of biomimetic scaffolding fabrics ranging from simple gauze or bandage materials to scaffolds for tissue culturing and a large variety of prostheses for permanent body implants.

Textile technologies are powerful tools for producing complex and hierarchical 3D constructs using bio-inspired fiber-based materials as building blocks. Textile platforms (weaving, twisting, braiding, and knitting) offer unique advantages, such as the versatility to fine-tune the properties of a scaffold size, shape, porosity, and mechanical properties by varying the assemble parameters—namely fiber diameter, fiber number, or braiding angle (Czaplewski et al. 2014)—with the potential to develop improved 3D constructs with biomimetic properties for achieving tenogenic differentiation (Czaplewski et al. 2014). Moreover, with textile technologies it is expected to grow in 3D and in a hierarchical architecture mimicking the native structure of the tendon.

A novel biofabrication modality, electrochemically aligned collagen (ELAC), allows the continuous production of aligned collagen threads through a pH gradient process between two parallel electrodes (Cheng et al. 2008) and combines textile techniques to fabricate complex 3D scaffolds. Since the development of this technique in 2008, several works report their potential for tendon and ligament-based approaches by mimicking the native tendon's structure and mechanical properties. ELAC scaffolds were shown to induce MSCs tenogenic differentiation (Kishore et al. 2012, Younesi et al. 2014) with the production of a tenomodulin positive matrix. Moreover, in a rabbit patellar tendon model, ELAC braided scaffold was shown to be biocompatible and biodegradable and assisted the increase in volume fraction of the tendon fascicle compared with the control (Kishore et al. 2012).

Although these fabrication technologies assist the alignment of collagen fibers and mimic tendon structure, the topographical and mechanical stimulation of cells is still limited in these scaffolds. Thus, the application of bioreactors to culture and stimulate biological processes in cells laden scaffolds may more effectively recreate tissue dynamic environment, improving the biofunctionality of these constructs aiming to mimic *in vivo* physiological conditions.

10.6 BIOREACTORS IN TISSUE ENGINEERING

10.6.1 Designing Bioreactors for Tendon Tissue Engineering

Bioreactors have been widely investigated as advanced tools for tissue engineering of musculoskeletal tissues. These dynamic systems are designed to provide different mechanical conditioning to cells or cell laden 3D matrices in order to resemble the physical forces experienced in the native tissue environment, such as shear stress, hydrostatic pressure, flow perfusion, microgravity or mechano-magnetic stimulation. For example, in bone tissues, nutrients and wastes are transported within a lacuno-canalicular network, whose circulation has been recreated by shear stress and flow perfusion systems (Rodrigues et al. 2012, Gardel et al. 2013). Bioreactors applying perfusion have also been shown to have promising results in cartilage studies (Rodrigues et al. 2012), as well as hydrostatic pressure bioreactors described to simulate the main forces to which cartilage is subjected to in the articular joints (Correia et al. 2012). Bioreactors offer controllable and reproducible dynamic environments with enhanced access of the cells to nutrient supply from the culture medium, improved oxygen diffusion and a more efficient metabolic waste *removal* (Table 10.1)**.** Moreover, bioreactors allow homogeneous and long-term cultures with the possibility for

TABLE 10.1
Summary of the Main Characteristics of a Bioreactor System

Cultures in Bioreactors

- Control over in vitro environment:
  - pH
  - temperature
  - humidity
  - oxygen tension
  - nutrient supply / waste removal
  - cell metabolite quantification
- Improved oxygen diffusion
- Homogeneous and long-term cultures (non-stop up to several months)
- Standardization of protocols
- Biochemical conditioning:
  - Single or multi dosage without interrupting with the experimental setup
- Biomechanical conditioning:
  - Stretching (mostly cyclic)
  - Loading
  - Tension/compression
  - Mechano-magnetic
- Closer environment to a tissue niche than static 3D cultures

Bioreactor components:

- Non-toxic, especially the parts in direct contact with the cultures
- Suitable for aseptic conditions
- Quick and easily assembled
- Sterilizable if reusable
- Preferably low-cost
- Easy to clean and store
- Portable, to fit in cell culture incubators if necessary
- Multiparameter/Tunability of parameters
- Computer control of parameters
- Real time monitoring using imaging techniques as microscopy, MRI or micro computed tomography
- Possibility for computer-assisted automation
- Possibility for scaling-up strategies

standardization and automation procedures. Depending on the system, external factors such as pH, temperature, and cell metabolite concentration can be monitored and adjusted, enabling higher cell proliferation rates and decreasing the number of cells that must be initially seeded, while favoring desirable cell responses. With the appropriate stimulus, it is also envisioned a reduction/elimination of medium supplementation including serum requirements. It is also expected that cultured cells subjected to the mechanical conditioning will be able to synthetize native tissue-like ECM in shorter periods of time and following a more controlled and organized distribution as the mechanical forces will likely better mimic the native niches. Thus, applying

bioreactors in tissue engineering strategies offers great advantages over 2D cultures and cell laden 3D matrices cultured in static conditions. These include (1) biomechanical conditioning of 3D constructs, providing adequate loading regimes according to the type of stimulation required; (2) increase of mass transport, as the supply of oxygen and soluble nutrients is a critical concern when culturing 3D constructs *in vitro*; (3) controlled culture conditions enabling the systematic study of tissue-specific physiological requirements; (4) computer monitoring and programmable options to control and adjust environmental parameters, reducing the limitations associated to a human operator; and (5) reproducible cycles of stimulation/standardization.

The development of bioengineered products is a time-consuming task and, thus, approaches to potentially accelerate their clinical use are needed. Bioreactor design can be more or less complex depending on the final application and monitoring parameters. However, all bioreactors are composed of a driving system, a control box and connection cables. The driving system is the motor or pump responsible for impelling mechanical stimulation or medium circulation through perfusion forces into the samples, often located in culture chambers. The control box or computer-aided software allows controlling the system, including fluid velocity and biochemical parameters. The tubing cables, often made of materials permeable to gases, are necessary to connect the different parts of the bioreactor to the power socket.

Most of the bioreactors for tissue engineering settings are designed to operate under aseptic conditions and inside a standard CO_2 incubator at 37°C. Thus, the assembly of the bioreactor and the positioning of the samples in the beginning of the experiment, as well as the handling of the system for medium exchange or collection of the samples for analyses are performed within biosafety hoods. The bioreactor materials should be non-toxic, especially the ones in contact with the constructs or tendon tissue samples, and bioreactor parts should be sterilizable if re-usable. Connecting parts such as tubes, nuts, o-rings, lids, and luer adaptors keep the system closed, and must be well tightened to prevent malfunctioning, fluid leakages and consequent contaminations. In order to assure gas interchange and pressure compensation, a 0.22 μm filter is normally used.

10.6.2 The Role of Bioreactors in Tendon/Ligament Tissue Engineering

The combination of multiple factors known to exist in tendon niches in a tridimensional and complex environment may enable the generation of predictive models relating to cellular responses towards scaffold design parameters and ultimately to recapitulate the alignment, the hierarchical architecture and tendon tissue formation. Commercial bioreactor systems for TTE have been designed to meet these requirements proposing a solution to the limitations of static cultures. Generally, these systems allow the researcher to control and manipulate the deformation cycles as well as the strain and rate levels applied on the sample. Hereafter, we will address some of these systems with different complexity that may be used for biologic tendon samples or TTE constructs.

10.6.2.1 Mechanical Stimulation

The bioreactors that have been used so far for TTE greatly differ in terms of complexity and multiparameter analysis (Figure 10.2).

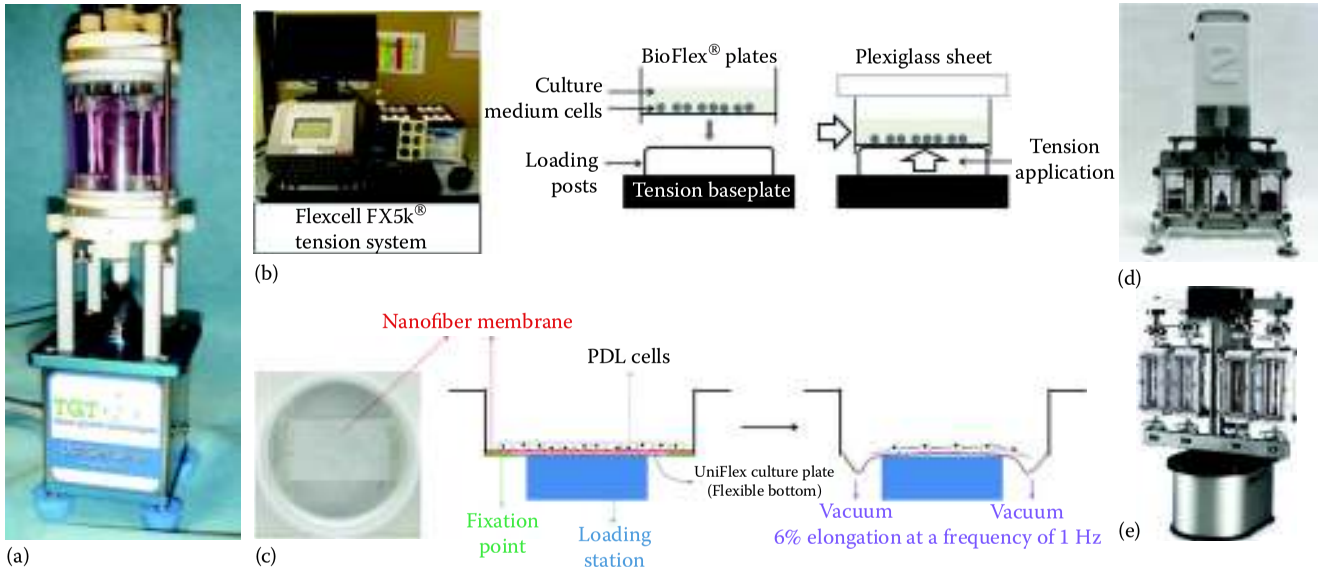

FIGURE 10.2 Images of some commercially available bioreactors used in TTE strategies. (a) LigaGen model L30-4C, DynaGen series, Tissue Growth Technologies. (Reprinted from *J. Hand Surg.,* 35A, Angelidis, I.K. et al., Tissue engineering of flexor tendons: The effect of a tissue bioreactor on adipoderived stem cell-seeded and fibroblast-seeded tendon constructs, 1466–1472, Copyright 2010, with permission from Elsevier.); (b) Schematic diagram of the FX5K® Tension System. (With kind permission from Springer Science+Business Media: *Knee Surg. Sports Traumatol. Arthros.,* Impact of cyclic mechanical stimulation on the expression of extracellular matrix proteins in human primary rotator cuff fibroblasts, 24, 2016, 3884–3891, Lohberger, B. et al.); (c) The Flexcell system equipped with the PDL cells supported on nanofiber matrix, where the dynamic mechanical tensional force was applied to the matrix/cell through equipment vacuum. (Reprinted with permission from Kim, J.H. et al., *PLoS One*, 11(3), e0149967, 2016); (d) TC-3 load bioreactor system, Reprinted with permission from EBERS Medical Technology SL, Cartuja Baja, Zaragoza); (e) ElectroForce® multi-specimen BioDynamic 5200. (Reprinted with permission from TA Instruments, New Castle, DE.)

One of the simplest systems is the Cell Stretching System from STREX, which was designed for stretching cells in culture and applying a stress load to cells up to 20% of stretching ratio. Morita and co-workers have been working with this device to investigate the optimal uniaxial cyclic stretching stimulation to bone marrow MSCs (Morita et al. 2013, 2014, 2015, 2017) towards tenogenic differentiation. This work suggest optimal normal strains between 7.9% and 8.5% for assisting the production of Col I and TNC proteins, respectively.

Flexcell International Corporation developed Flexcell® Tension Systems, which are computer-regulated bioreactors that use vacuum pressure to apply cyclic or static strain to cells growing *in vitro* with control over the magnitude and frequency of the stretching. Depending on the model, some systems can check and analyze real-time cellular biochemical changes in response to strain. In a work developed by Kim and colleagues (Kim et al. 2016), the FX-5000 tension system from Flexcell® was used to apply mechanical stress (strain of 6% elongation at a frequency of 1Hz) to rat periodontal ligament (PDL) cells seeded onto nanofiber-equipped culture plates with random or aligned topography. The cells cultured on the oriented nanofibers combined with the mechanical stress produced PDL specific markers, including periostin and TNC, undergoing ligamentogenesis with simultaneous down-regulation of osteogenesis. Moreover, the cell/nanofiber constructs engineered under mechanical stress showed sound integration into tissue defects with significantly enhanced new bone volume and area, in a rat premaxillary periodontal defect model (Kim et al. 2016). In another recent study from Sun and co-workers (Sun et al. 2016), rabbit fibroblasts from ligament tissues and bone marrow MSCs were mechanically tested under Uniflex/Bioflex culture system from Flexcell®, as a mean to mimic mechanical strain in ligament tissue. Results showed that uniaxial stretch (15% at 0.5 Hz; 10% at 1.0 Hz) stimulated fibroblast proliferation and collagen production, while uniaxial strains (5%, 10%, and 15%) at 0.5 Hz and 10% strain at 1.0 Hz were favorable for MSCs. Similar results on the increment production of total collagen by human fibroblasts from the rotator cuff with cyclic strain (Flexcell FX5K™ Tension System; 10% elongation and 0.5 Hz frequency) were achieved by Lohberger and colleagues (Lohberger et al. 2016). Also, increasing levels of the matrix metalloproteinases MMP1, MMP3, MMP13, and MMP14, analyzed by RT-qPCR, were observed in stimulated conditions as well as tenascin-C and scleraxis.

The LigaGen® Ligament and Tendon Bioreactor from BISS TGT was also designed to provide mechanical stimulation, imposing axial stress or strain to 3D tissue engineered constructs or decellularized tissues to recreate physiological conditions *in vitro*, with studies aiming at hand tendons and anterior cruciate ligament (ACL) regenerative medicine. The chambers of this bioreactor deliver oscillatory axial stimulation to the samples. The stress/strain profiles are defined by the operator, which can be in the form of a simple harmonic (sinusoidal) or a physiological waveform. The bioreactor can be complemented with a perfusion system to provide convective media transport around the samples.

The TC-3 load bioreactor from EBERS Medical Technology SL is a computer-controlled system designed to enclose tissue samples or cell laden scaffolds under mechanical tension and compression axial loading.

Herein, the tension grips apply tension loads on samples as different as sheet-like, membrane substrates or 3D-like samples. This specific bioreactor can operate in two different working modes, horizontal or vertical configuration, depending on the type of experiment to be developed as the requirement for immersion or air liquid interface, for example. TC-3 load bioreactor allows simultaneous flow and deformation conditions, but hydrostatic pressure conditions can also be simulated.

ElectroForce® BioDynamic® systems from TA Instruments can be used to simulate *in vivo* conditions and provide accurate characterization of biomaterials and biological specimens under tension and perfusion flow regimes. Also, an integrated digital video extensometer can be added to the system for primary, secondary, and shear strain measurements. Ligaments, tendons or other thin and elongated specimens are attached to the tensile grips while the chamber is perfused with nutrients. The great advantage of this bioreactor in comparison to the above described is the AQ 10
possibility of integrating a Dynamic Mechanical Analysis (DMA) software, allowing determination of the viscoelastic properties at the same time of culture/stimulation.

Apart from the model used, the significant cost of commercial bioreactors and the limited number of samples the operator can handle per experimental setup are the main disadvantages pointed out. Therefore, several research groups have custom designed bioreactors, developing new systems to meet more accurately the specific parameters of a tissue or tissue substitute to be screened and evaluated.

One of the most relevant parameters for tendon substitute development is the application of cyclic strain (Screen et al. 2005, Doroski et al. 2010, Andarawis-Puri et al. 2012, Legerlotz et al. 2013), and thus a major consideration to the customization of bioreactors.

Wang and colleagues (Wang et al. 2013) developed a bioreactor system, which applies pre-programmed uniaxial stimulation, to study different cyclic tensile strain (0.25 Hz for 8 hours/day, 0%–9% for six days) on rabbit Achilles tendons. Overall results showed that 3% cyclic tensile strain did not prevent matrix deterioration (gene expression of MMP1, 3, and 12 were highly upregulated by 3% strain stimulation compared to the other groups), whilst at 6% cyclic tensile strain the structural integrity and cellular function of the tendons were maintained. Moreover, at 9%, massive rupture of the collagen bundles was also verified.

Youngstrom and co-workers (Youngstrom et al. 2015) studied the influence of cyclic mechanical conditioning (0%, 3%, or 5% strain at 0.33 Hz for up to 1 hour daily for 11 days) provided by a custom bioreactor on the maturation and cellular phenotype of decellularized tendons obtained from four equine sources seeded with bone marrow-derived MSC. Cultured cells at 3% and 0.33 Hz integrated within these tissue-derived scaffolds, exhibited higher elastic modulus and higher expression of tenogenic genes.

10.6.2.2 Magnetic Stimulation

In recent years, magnetic driven actuation has been investigated as an alternative form of bio-stimulation in TE strategies. It is known that magnetic forces influence biological processes, and magnetotherapy protocols have been proposed in tissue regeneration and inflammation control after injury. Furthermore, magnetic stimulus may act synergistically with magnetizable nanoparticles internalized by cells in

culture or embedded within 3D scaffolds creating local forces, which can be physically sensed by cells assisting mechanotransduction processes that will ultimately lead to an *in vitro* maturation of the cell-laden constructs prior to implantation. This approach has been previously hypothesized and reported by our group on the use of magnetic bioreactors in the stimulation of stem cells towards tenogenic, osteogenic, or chondrogenic differentiation (Lima et al. 2015, Goncalves et al. 2016, Santos et al. 2016) and by others for osteogenesis (Meng et al. 2013, Kang et al. 2013), cardiac TE (Sapir et al. 2014), and neuronal regeneration (Antman-Passig and Shefi 2016).

3D-printed magnetic scaffolds cultured with hASCs exposed to oscillation frequency conditions provided by a magnefect-nano transfection device (nanoTherics Ltd, UK) showed that magnetic stimulation tend to accelerate the production of collagen and noncollagenous proteins by cells after seven days (Goncalves et al. 2016). This device was initially set up for magnetofection purposes, but the magnetic properties of the system showed potential for applications in magnetic force-based TE.

On the other hand, magnetic responsive membranes, which were implanted subcutaneously in rats exposed to a pulsed electro-magnetic field (PEMF) waveform with a magnetic field intensity peak of 0.01 T, a duty cycle of 6.3 ms and a frequency of 75 Hz for 2 hours a day, five days a week (Magnum XL Pro, Globus), showed to modulate tissue inflammatory response, translated by a decrease in the number of mast cells infiltration and reduction of the thickness of the fibrous capsule (Santos et al. 2016). The coils that provided the mechano-magnetic stimulation within a therapeutic mat were placed under the animals' cage. Magnum devices from Globus are commercially available magnetotherapy devices that provide low-frequency pulsed magnetic fields, being composed of solenoids that permit both the superimposition and the opposition of the magnetic field to treat surface or deep pathologies. These instruments are generally used in human clinical procedures, mostly physiotherapy centers, for applications in muscle, bone-tendon, and anti-ageing treatments. The use of magnetic forces in tissue healing is quite recent and some pioneer studies suggest the influence of magnetic fields in modulating tendon injury recovery after rat Achilles transection (Strauch et al. 2006). Besides pain relief (Nelson eta l. 2013) and stimulation of blood circulation, magnetotherapy has been reported to stimulate tendon cell proliferation (Seeliger et al. 2014, Randelli et al. 2016) in the promotion of the healing process.

Bioreactors that generate PEMF have also been investigated. Recently, Liu and colleagues (Liu et al. 2016) and Tucker and colleagues (Tucker et al. 2017) described the use of a commercial device, Physio-Stim® PEMF system from Orthofix Inc, to promote gene expression of human tenocytes (collagen I, TGFβ-1, PDGFβ, BMP12, and TIMP4) and to improve early tendon healing in a rat rotator cuff model, respectively. The FDA approved Orthofix stimulators claim to generate a uniform, low-level PEMF shown to be safe in clinical studies for the healing of nonunion fractures (Garland et al. 1991).

The portable SomaPulse® is another non-invasive PEMF system. It applies a sequence of magnetic pulses programmed to introduce a magnetic field into musculoskeletal tissues. Despite the multiple devices available for magnetic stimulation, the application of electromagnetic fields is still not properly understood, nor how the exposure to PEMF influences tendon resident cells or tendon tissue responses.

Despite the recent scientific interest on the magnetic force impact over biological tissues, the wide range of magnetic properties, such as intensity, time of exposure or frequency, has to be more deeply explored and optimized to individual conditions, tendon anatomical location, and associated pathologies. Electromagnetic fields are expected to influence cells response at the molecular levels or to act as mediators of inflammation. Girolamo and co-workers (Girolamo et al. 2013) reported that a PEMF (1.5mT, 75 Hz) enhanced tendon cell proliferation and the release of anti-inflammatory cytokines and angiogenic factors (IL-1B, IL-6, IL-8, and TGF-β). Herein, the PEMF was generated by a pair of rectangular horizontal coils placed opposite to each other.

10.7 MECHANOREGULATION MECHANISMS

Physiological responses to mechanical loading are initiated by a process called mechanotransduction, in which cells detect physical changes in their microenvironment through specialized machinery and then translate that information into an appropriate biological response (Santos et al. 2015). This mechanosensitive feedback mechanism modulates cellular functions as proliferation, differentiation, migration, and apoptosis, and is crucial for organ development and homeostasis (DuFort et al. 2011).

Tendon tissues, physiologically adapted to transmit mechanical forces in a daily basis, are the perfect model to study the mechanisms involved in the translation of mechanical forces into a functional response.

Growing evidence suggests that mechanical forces regulate the expression of the bHLH transcription factor *Scx* through activation of the TGF-β/Smad2/3 pathway AQ 11
in adult tenocyte cultures, which, in turn, is required for maintenance of tendon-specific ECM (Maeda et al. 2011, Havis et al. 2016, Gaut and Duprez 2016).

Furthermore, it is accepted that these forces can be at least partially mimicked by the stimuli provided by bioreactors.

Mohawk (*Mkx*) and the downstream tendon-associated genes Tenomodulin (Tnmd), Collagen type I (*Col1a1* and *Col1a2*), but not Scleraxis (*Scx*), showed an increased expression in Achilles tendon-derived rat tenocytes subjected to stretching at 2% and 0.25 Hz for 6 hours in a FX-5000 tension system (Flexcell International) (Kayama et al. 2016).

Moreover, magnetic-mechano actuation directed to cell surface receptors is a good example to remotely deliver mechanical stimuli into individual cells. Studies reported that a magnetic field of variable frequency may influence cellular response and intracellular signaling favoring the differentiation into desired phenotypes and higher proliferation rates in a shorter culture time and in a more reproducible manner (Girolamo et al. 2013, Henstock et al. 2014, Rotherham and El Haj 2015, Sapir-Lekhovitser et al. 2016).

3D scaffolds may also be used for tendon mechanobiology studies, in which the actuation of mechanical loads provided by bioreactors may be combined with the stimulation from the topographical and physicochemical properties of the scaffold to the seeded cells. An example is the fiber composite hydrogels developed by Screen and co-workers (Screen et al. 2010) who envisioned to be a mechanotransduction

research platform. Collagen type I gene expression was upregulated in NIH/3T3 fibroblasts laden in the hydrogels and subjected to cyclic tensile loading of 5% dynamic tensile strain, at 1 Hz for 24 hours.

Also, Jones and colleagues (Jones et al. 2013) showed that TGFβ activation plays an important role in mechanotransduction, specifically in the regulation of MMP genes of human tenocytes isolated from tissues with tendinopathic conditions. These tenocytes were seeded onto 3D collagen gels and a 5% cyclic uniaxial strain at 1 Hz for 48 hours was applied over these constructs using the Flexcell FX-4000™ device. Treatments with TGFβ1/TGFβRI inhibitor were compared to mechanical strain regimes, and the outcomes with strain or TGFβ treatment were similar. Overall, there was a decrease in MMP1, -3, -11, -13, and -17 and an increase in collagen type I at the mRNA level (Jones et al. 2013).

10.8 CONCLUDING REMARKS

Since the first investigation of tendon/ligament bioreactors published in the 1990s (Hannafin et al. 1995), these devices have evolved into more complex systems able to test more specimens simultaneously and control/program several parameters. Despite the advances in recent years and the awareness for mimicking the different fundamental aspects of tendons, which are intrinsically associated to tissue function and activity, currently available tendon substitutes are not biomechanically competent as artificial replacements of tendons. Nevertheless, bioreactors can fulfill this functional gap offering a powerful solution for improving and assisting the development of new tissue engineering equivalents as they provide a controlled, dynamic and monitorable environment that more closely resembles native tissues, with potential toward scale up strategies.

Moreover, bioreactors provide the possibility for testing a variety of different cell laden 3D structures, including scaffolds, membranes, tissue explants, and more, that can be investigated and assessed in systematic and reproducible conditions as predictive tools of tissue substitute performance in similar physiological conditions, resembling the native environment. However, the optimal *in vitro* conditions and the optimal 3D tissue substitute have not been established, and the challenge stands for an accurate time spatial recapitulation of physical and biochemical signals that cells may experience in tendon niches, as well as cell response to such potential stimuli, providing important insights into the long-term capability of engineered constructs to maintain tissue proper functionality. Cellular mechano-sensing mechanisms and the information exchange in biomechanical regulatory signals between the cell and its surroundings also have an important role in determining the potential outcomes of bioreactor microenvironments towards pre-clinical models. These issues need to be thoroughly addressed in forthcoming years in order to achieve bioreactor designs that fully comprise biological and biomechanical demands of tendon tissue.

AQ 12 **LIST OF ABBREVIATIONS**

2D Two dimensional
3D Three dimensional
ACL Anterior cruciate ligament

BGN	Byglican
BMP	Bone morphogenetic protein
Col I	Collagen type I
DCN	Decorin
DMA	Dynamic Mechanical Analysis
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELAC	Electrochemically aligned collagen
FACITs	Fibril-Associated Collagens with Interrupted Triple-helices
FGF	Fibroblast growth factor
GDF-5	Growth and differentiation factor 5
hASCs	Human adipose-derived stem cells
Hz	Hertz
IGF-1	Insulin-like growth factor 1
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem/stromal cells
MMPs	Matrix metalloproteinases
Mkx	Mohawk
PCL	Poly(ε-caprolactone)
PDGF	Platelet-derived growth factor
PDL	Periodontal ligament
PEMF	Pulsed Electromagnetic Fields
SPCL	Starch poly(ε-caprolactone)
TE	Tissue engineering
TIMP	Tissue inhibitor of metalloproteinases
TSPCs	Tendon stem/progenitor cells
TTE	Tendon Tissue Engineering
TNC	Tenascin
TGF-β	Transforming growth factor beta
Scx	Scleraxis

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